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GRANT NUMBER DAMD17-93-J-3032

TITLE: A Genetic Analysis of NF1 Function in Drosophila Melanogaster (Neurofibromatosis)

PRINCIPAL INVESTIGATOR: Iswar Hariharan, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, Massachusetts 02114

REPORT DATE: October 1996

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 4

REPORT DOCUMENTATION PAGE

Form Approved
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Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1996	Final (22 Sep 93 - 21 Sep 96)

4. TITLE AND SUBTITLE A Genetic Analysis of NF1 Function in Drosophila Melanogaster (Neurofibromatosis)

5. FUNDING NUMBERS
DAMD17-93-J-3032

6. AUTHOR(S)
Iswar Hariharan, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts General Hospital
Boston, Massachusetts 02114

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The human neurofibromatosis type 1 (NF1) protein contains a RasGAP domain and is believed to restrain cell proliferation by negatively regulating Ras-mediated signalling. We have characterized a *Drosophila NF1* homologue encoding a protein that is 60% identical to the human NF1 protein over its entire length and generated loss-of-function mutations. Flies lacking *NF1* are viable and *Ras1*-mediated signalling downstream of the *sevenless* and *torso* receptor tyrosine kinases is normal, indicating that *Drosophila NF1* is not a crucial *Ras1* regulator. However, combined loss of *NF1* and another *Drosophila* RasGAP, *Gap1*, is lethal, arguing that *NF1* may be a redundant *Ras1* regulator *in vivo*. Mutants lacking *NF1* are 25-30% smaller than wild type animals during all post-embryonic developmental stages and mutants also display a diminished escape response. Remarkably, the size defect is not modified by manipulating *Ras1* pathway signalling but is rescued by providing increased levels of PKA activity. Thus *NF1* may modulate both PKA and Ras signalling *in vivo* and could conceivably provide a link between these signalling pathways.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Neurofibromatosis, Genetics, Drosophila Melanogaster, NF1, Mutagenesis	21
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Susan Heisler 10/19/96

PI - Signature Date

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FINAL REPORT

INTRODUCTION

Neurofibromatosis type 1 (*NF1*) is one of the commonest human genetic diseases. Although the disease causes significant morbidity and mortality by predisposing affected individuals to benign and malignant tumors, the pathogenesis of the disease is poorly undestood and no cure exists. The gene mutated in *NF1* patients encodes a large protein which has domains with sequence similarity to mammalian rasGAP and the yeast IRA1 and IRA2 proteins (reviewed in (1). Although the *NF1* protein can function as a rasGAP in vitro, the normal biological function of *NF1* and its precise role in the pathogenesis of the disease are poorly understood.

In order to study the normal function of *NF1*, we chose to utilize a genetic approach. Prior to the commencement of this proposal, we had cloned a highly conserved *NF1* homologue from *Drosophila melanogaster*. This report details our analysis of the expression and function of Drosophila *NF1* using mutants that we have generated.

As outlined in the Statement of Work in our original proposal, the objectives were as follows:
Year 1 - Characterization of *NF1* RNA and protein expression and a screen for lethal mutations in the vicinity of *NF1*. This objective has been completed. We found however, that none of the lethal mutations were lethal. We therefore needed to use an alternate approach.
Year 2 - During this year we conducted (contingency plan in our original SOW) a P-element screen to isolate *NF1* mutations. This approach was successful. Mutations in *NF1* were not lethal but demonstrated a surprising requirement for normal growth and neuromuscular function.
Year 3 - As stated in our original SOW, we completed phenotypic analysis of the mutants including clonal analysis. In our original SOW, we had planned to identify interacting genes and to map functional domains in the *NF1* protein. However, when we found a novel link between *NF1* and the PKA signaling pathway, we pursued this interaction in more detail instead. This work has led to defining a completely novel function for *NF1* and a means of suppressing defects of *NF1* mutants in vivo.

These results are described in this report. These results have been included in two papers which have been submitted to Nature and are currently being reviewed.

1) The, I., Hannigan, G. E., Reginald, S., Zhong, Y., Gusella, J. F., Hariharan, I. K. and Bernards, A. Role for Drosophila *NF1* in growth regulation and PKA-mediated signalling.
2) Guo, H-F., The, I., Hannan, F., Hariharan, I. K., Bernards, A and Zhong, Y. neurofibromin regulated signaling in Drosophila neuropeptide transmission: adenylyl cyclase and ras pathways.

BODY

EXPERIMENTAL METHODS

Molecular biology

Most DNA, RNA and protein manipulations involved standard procedures Genomic clones were isolated by screening a λFIX-II *Drosophila melanogaster* Canton S

genomic library (Stratagene) with a probe representing the C-terminal 1598 codons of the human *NF1* reading frame. The hybridization was performed at 37^0 C in buffer containing 5 x SSC, 25% formamide, 5% Dextran sulfate, 5 x Denhardt's solution, 0.5% SDS, and 100 mg/ml denatured DNA. Filters were washed 6 times for 5 min in 3 x SSC at room temperature and exposed for 3 days. Drosophila *NF1* cDNAs were isolated from eye disc, total disc and mixed stage embryo libraries. DNA sequences were determined by the dideoxy chain termination method, using Sequenase enzyme (U. S. Biochemical).

Drosophila embryos were collected on molasses-agar egg laying plates, dechorionated with 50% bleach, dounced in a buffer containing 20mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and centrifuged for 20 min at 14,000 x*g* at 4^0 C to remove debris. Equal amounts (100-150 mg/lane) of total protein was size separated by SDS polyacrylamide gel electrophoresis and tranferred to immunoblots. The latter were probed with anti Drosophila *NF1* monoclonal antibodies *NF1*-11 or *NF1*-21, which had previously been generated by the MGH Cancer Center Monoclonal Antibody Core Facility. *NF1* was precipitated from embryo lysates using a cocktail of four monoclonal antibodies (*NF1*-11, 21, 30 and 33).

Genetics

Flies were raised on cornmeal-molasses-agar-yeast medium and all crosses were carried out at 25^0 C. Strains, mutations and balancer chromosomes are described in (2).

F_2-lethal screen

Isogenized *bw; st e* adult males were starved for 1 hr. and given 25 mM ethyl methane sulfonate (EMS) in 1% sucrose for 16 hr, transferred to a vial with adsorbent tissues for 1 hr, and mated to *bw*; *TM3/TM6B* virgin females. Flies were allowed to lay eggs for 24 hr and were then transferred into new bottles. After five days the parents were discarded. Resulting F_1 *bw; st e */TM3* or *TM6B* males were individually crossed to bw; *Df(3R)Df(3R)boss*^{16} *st e/TM3* or *TM6B* virgin females (* indicates the mutagenized chromosome). Since adults homozygous for both the *brown* (*bw)* and *scarlet* (*st*) pigment genes have white eyes, the absence of white eyed flies in the F_2 progeny indicates the presence of a mutation in the st e* chromosome that is lethal in trans to the *Df(3R)boss*^{16} *st e* chromosome. The mutagenized chromosome was recovered from siblings of the genotype *bw; st e */TM3* or *TM6B*.

Recombination mapping:

The multiply-marked third chromosome *th st cu sr e ca* stock was used to map complementation group *l(3)D*. Males of *l(3)D/TM3* were mated with virgin females *th st cu sr e ca*. Males *th st cu sr e ca* were crossed with virgin females *l(3)D / th st cu sr e ca* in which the recombination occurs. Since all flies from this complementation group displayed a dominant wing vein phenotype, the progeny were scored for linkage of the wing vein phenotype with one of the markers.

To map group *l(3)A* the stock was crossed into a white background. Males of *w; l(3)A/TM3* were crossed to red eyed virgin females *w; P[w].* Males *w; TM3/TM6B* were crossed to virgin females *l(3)A/P[w].* The red eyed progeny of this cross were then tested for lethality in combination with other alleles of the *l(3)A* complementation group. When the lethal mutation is tightly linked to the *P[w],* hardly any recombination events would occur between the lethal mutation and the *P[w]* . The number of progeny from red eyed flies which is lethal with *l(3)A* would be small.

P-element screen

To generate specific*NF1* mutants *w; P[w]* males homozygous for a *P[w]* inserted in 96F were crossed to virgin females *Ki p*^{p} *D 2-3* bearing a transposase. Single F_1

dysgenic males with the phenotype *P[lacZ w]/Ki* p^p *D 2-3* were crossed to virgin females *w*; TM3/TM6B. Single F_2 males w; *P[lacZw]*/TM3 or TM6B were crossed to female virgins *w*; TM3/TM6B to establish single lines with stable novel P-element integrations. The red eyed progeny of this cross was analyzed in pools by inverse PCR.

Inverse PCR screen for *de novo* P element integrations in *NF1*.

Genomic DNA was prepared from pools of 40 red-eyed flies by douncing in 0.1 M Tris-HCl pH 8.5, 0.1 M EDTA, 0.1 M NaCl, and 0.5% SDS. Before homogenization, a single homozygous K33 mutant was added as a positive control. 20 ml of DNA was digested with 4 units of Sau3A for 3 hr. After 1:1 phenol:chloroform extraction and ethanol precipitation, DNA pellets were resuspended in 50 ml TE buffer. The digested DNA was then circularized by ligating 5ml in a 200 ml volume with 40 units T4 DNA ligase (New England Biolabs) at 16^0 C for 12-16 hr. The ligated DNA was phenol:cloroform extracted, ethanol precipitated, and resuspended in 10 ml of water. The circular products of the ligation reaction were used as a template for PCR (5 min. 85^0 C, followed by 40 cycles of 1 min. at 94^0 C, 1 min. at 58^0 C, 2 min. at 72^0 C), using a Perkin Elmer-Cetus thermal cycler. Reactions contained 10 ml of ligated DNA in a 20 ml total volume with final concentrations of 0.2 mM dNTPs (Boehringer Mannheim), 1 x PCR buffer (Promega), 1.25 mM Mg Cl2, 125 ng of primers, 1 unit of Taq polymerase (Promega) and 10 mCi [a-^{32}P]dCTP (NEN). The primers used were: CGA CGG GAC CAC CTT ATG TTA TTT CAT CAT G (P-element inverted repeat); GCC GAA GCT TAC CGA AGT ATA CAC T (5' end); GCA AGA GAC ATC CAC TTA ACG TAT GC (3' end).

DNA of phage lE8, which contains the entire *NF1* gene, was digested with EcoRI. Similarly digested R5.17 phage DNA, which spans the integration site of the parental P-element, was included as a positive control. Hybridization of PCR-generated probes was allowed to proceed at 65^0 C for 12-16 hr in 5 x SSC, 5 x Denhardt's solution, 50% formamide, 0.5% SDS, and 150 mg/ml denatured DNA. Membranes were washed at 65^0 C for 2 hours with four changes of 2 x SSC, 0.1% SDS. Autoradiography was carried out for 1-5 days at -80^0 C using Kodak X-omat film and intensifying screens.

Clonal analysis

Clonal analysis was performed in a *forked (f)* background. Females *f; bld P[f+]/TM3 Ser* were crossed to males $NF1^{P1}$, $NF1^{P2}$ or the parental K33 strain. Parents were transferred after 24 hr and the larval progeny irradiated after 48-72 hr. Adult F1 males *f; bld P[f+]/* $NF1^{P1}$ or $NF1^{P2}$ or K33 were analyzed for presence of clones in the wing and the wings were mounted in Canada balsam (Sigma). The clones were investigated with a compound microscope and 32x magnified clones were photographed The distance of the bristles in the *forked NF1* mutant clone were measured and compared to the distance of the bristles in the surrounding heterozygous tissue.

Generation of *hsp70-NF1* transgenic flies

The mammalian *NF1* open reading frame is strongly se;lected against in E. coli. To avoid similar toxicity problems, we constructed an *hsp70-NF1* mini gene by cloning a hybrid cDNA/genomic *NF1* insert into the SacII and KpnI sites of the pKB176PL P element-based vector (3). Expression of this mini-gene in E. coli is prevented by the presence of three introns in the genomic part of the insert (flanked by unique MluI to Esp3I sites). The resulting P element was introduced into the germline of w^{1118} flies by injection. Administering daily 30 min 37^0 C heat shocks fully rescued the *NF1* deficient phenotype.

In situ hybridization to polytene chromosome

Salivary glands were obtained from wandering third instar Canton-S larvae and polytene chromosomes were prepared for hybridization. Two non-overlapping genomic fragments were used as probes. Probes were labeled with biotin-11-dUTP by nick-translation and hybridization was performed as described (4).

RNA in situ hybridization

Digoxigenin-labeled sense and antisense *NF1* and *evenskipped* RNA probes were made from linearized cDNAs using T3 or T7 RNA polymerase respectively. *In situ* hybridization to whole mount embryos was performed essentially as described (5), except that 4 mg/ml of proteinase K was used.

Embryo and imaginal disc staining

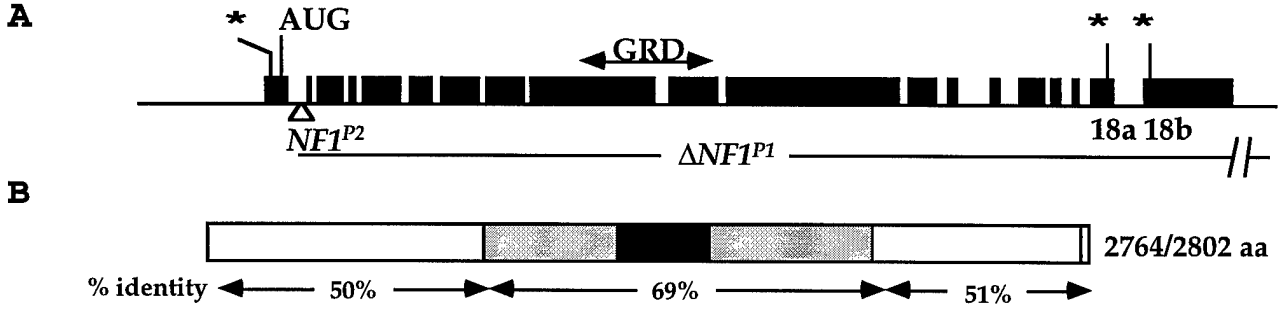
Embryo and imaginal disc staining was performed as described (6).

Phenotypic analysis

Scanning electron microscopy of adult Drosophila eyes and retinal sections were prepared as described (11). Wings were dehydrated in 100% ethanol and mounted in Canada balsam (Sigma).

RESULTS

A *Drosophila NF1* homologue was identified by screening a genomic library with a human *NF1* cDNA probe. Restriction mapping demonstrated that several independent clones were all derived from the same locus. A 13,295 bp region encompassing the entire homologue was fully sequenced. We also determined 9750 bp of overlapping cDNA sequence. Alignment of the genomic and cDNA sequences shows that *Drosophila NF1* consists of 17 constitutive and 2 alternatively spliced exons 18a and 18b (Figure 1A). The 2764 and 2802 amino acid proteins predicted by the alternatively spliced cDNAs are 60% identical to the human *NF1* protein, called neurofibromin (Figure 1B and C). Sequence similarity is observed over the entire length of the protein, including regions that fall outside the GAP catalytic domain and the more extensive segment related to yeast IRA1&2 proteins. Moreover, 11 out of 17 *Drosophila NF1* splice sites map within two codons of splice sites in the human gene. No other *NF1*-related sequences were detected during low stringency screens of cDNA or genomic libraries, suggesting that this gene is the only *Drosophila NF1* homologue.

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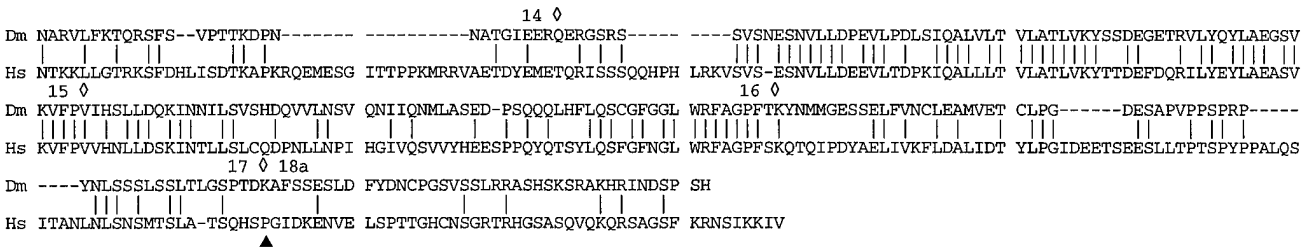

Figure 1. *Drosophila NF1* gene structure and comparison of the encoded protein to human neurofibromin. The top drawing (A) shows the intron-exon structure of *Drosophila NF1* and the location of translational start and in-frame stop codons. The $NF1^{P2}$ allele harbors a *P*-element at the indicated position in the first intron. The extent of the deleted segment in $NF1^{P1}$ is also indicated. Drawing B shows the percentage amino acid sequence identity between the indicated segments of *Drosophila* and human *NF1*. The GAP-related domain (GRD) and IRA-related segments are drawn as black and shaded boxes, respectively. The sequence alignment (C) compares the *Drosophila* (Dm) and human (Hs) proteins. Dashes were introduced to optimize the alignment. Amino acids encoded by the last complete codon in each exon are identified by signs. Exon numbers are indicated to the left of splice junctions. The boxed segment shows the approximate extent of the GRD. Three positions downstream of which alternatively spliced exons insert short amino acid segments in human neurofibromin, are identified by filled-in triangles. One of these locations corresponds exactly to the position where *Drosophila* exon 17 is joined to either exon 18a or 18b. Exon 18b includes a translational terminator after a single codon and cDNAs harboring this exon predict a 2764 residue protein ending in PTDKAA.

Expression of *Drosophila NF1* RNA and protein

For many genes, analysis of the spatial and temporal patterns of expression has provided clues as to the function of the gene. For instance, the *Drosophila Gap1* gene, which encodes another RasGAP, is expressed in specific regions of the imaginal discs and the patterning of these same regions is perturbed in *Gap1* mutants (7). We therefore examined whether *NF1* was expressed at higher levels at particular stages of development or in particular tissues. A 9.5 kb mRNA was seen in all developmental stages, but this transcript was especially prominent in adult flies and in early (0-6 hr) embryos (Figure 2). Hybridization with a *Ras2* probe (8), which detects several transcripts that are expressed at similar levels throughout development, showed that all lanes were equally loaded. Since little transcription occurs during the first few hours post-fertilization (9), the earliest mRNA presumably represents maternal transcripts. A less abundant 10.5 kb mRNA appears in 3-24 hr embryos and may be an alternatively spliced species. We conclude that *NF1* is expressed at most stages of development.

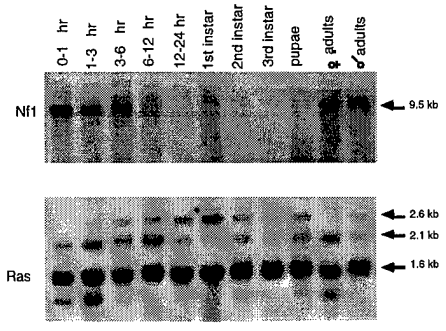

Fig. 2. Expression of *Drosophila NF1* is regulated during development. Each lane of the RNA blot contains 3 mg of poly(A) RNA from staged embryos, larvae, pupae, and adults as

indicated The top panel was hybridized to a *NF1* probe. The bottom panel shows the same blot after rehybridization with a *Ras2* probe.

To analyze the spatial distribution of *NF1* expression, we performed *in situ* hybridization of whole-mount embryos with digoxygenin labeled RNA probes. Under conditions where *even-skipped* (10) (10), *Rapgap1* (our unpublished data), and *Rac1* (11) (11) probes showed the expected patterns , *NF1* probes showed uniform staining of all embryonic tissues. This result was obtained with two non-overlapping probes from the coding region. No staining was observed with the control sense-strand probe. This indicates that *NF1* transcripts are present in most, if not all, cell types.

Monoclonal antibodies recognizing *NF1* had been previously generated by the MGH monoclonal antibody facility. We have used these antibodies to stain embryos and tissues from third instar larvae and adult flies. *NF1* is expressed at low to moderate levels in most, if not all cell types but is expressed at somewhat higher levels in the proliferative centers of the larval brain and in the ovaries. Flies that express no *NF1* protein (see below) were used as controls and show no staining under the same experimental conditions. Since *NF1* is widely expressed, it is likely that it has a function that is relevant to most cell types.

Generation of mutants in *Drosophila NF1*

The most direct way of testing the function of a gene is to generate mutations that disrupt its function. Additional information can often be obtained by examining the phenotypic consequences of increasing the activity or the levels of expression of a gene product. We have utilized both of these approaches to study the function of *Drosophila NF1 in vivo*.

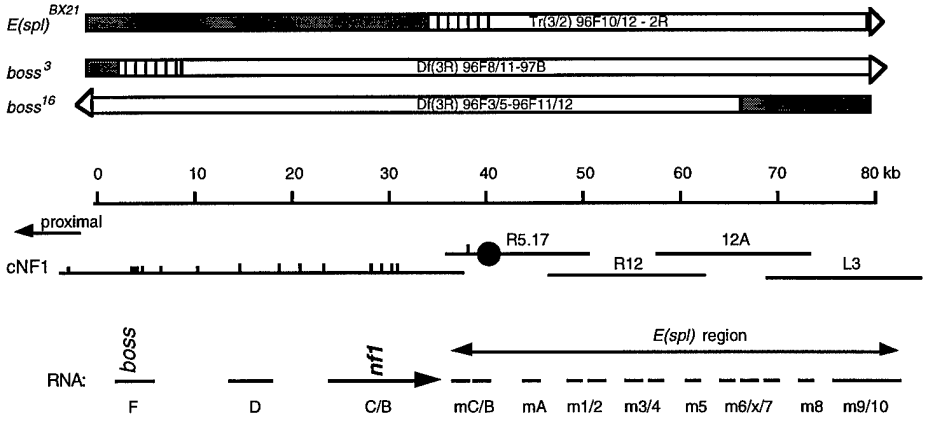

Fig 3. Map of the 96F region showing the approximate extent of *Df(3R)boss*^{3} and *Df(3R)boss*^{16} deficiencies and the location of the *T(2,3)E(spl)*^{BX21} translocation. In the schematized chromosomes filled boxes indicate intact DNA, hatched boxes regions harboring rearrangements, and open boxes deleted segments. The transcription map shows the location of *boss,* an unidentified transcript *D* (12), *NF1*, and *E(spl)*. The filled circle indicates the location of a *P* element in the K33 strain. c*NF1* is a cosmid; R5.17, R12, 12A, and L3 are phage clones.

In order to generate loss-of-function mutations in *NF1*, we first determined the chromosomal location of *NF1*. *In situ* hybridization of salivary gland chromosomes mapped *Drosophila NF1* to cytogenetic interval 96F-97A on the right arm of the third

chromosome. Since DNA spanning this entire region has been cloned, we mapped cosmid clones from the region and were able to determine the precise location of *NF1* with respect to transcription units in the region. The *Drosophila NF1* gene (Figure 3) is located between the *bride of sevenless* gene (12), and the *Enhancer of split* gene complex (13).

Our initial approach to obtaining loss-of-function mutations in *Drosophila NF1* was based on the assumption that *NF1* gene function was required for viability. This seemed likely since homozygous loss of murine *NF1* is lethal, and because any combination of deletions that removed both copies of the *NF1* gene was also lethal. We therefore screened the 96F region for lethal mutations that were uncovered by the *Df(3R)boss*^{16} chromosome (Figure 4) using chemical mutagenesis. After screening 13,500 flies we isolated 60 lethal or semi-lethal mutations in 6 complementation groups. None of these complementation groups comprised mutations in *NF1*. We have since confirmed that loss-of-function mutations in *NF1* are indeed not lethal (see below).

Since the results of our screen suggested that mutations in *NF1* did not cause lethality, we chose an alternate strategy which made no assumptions about the nature of the mutant phenotype. We chose to generate mutations by "local hopping" of *P*-element transposons. We identified a line (K33), which harbored a *P[white]* element approximately 25 kb downstream of *NF1* (Figure 3). To generate hops into *NF1*, this transposable element was mobilized by crossing these flies to a line containing a stable source of transposase. Following the scheme shown in Figure 4, lines of flies which contained putative local hops were set up, DNA was prepared from their progeny and screened using a variation of the polymerase chain reaction (inverse PCR) to detect transpositions into the immediate vicinity of the *NF1* gene (14). Of 1600 lines screened, seven had novel integrations in the immediate vicinity of *NF1*. Five of the integrations occurred 3' to the coding region while two integrations disrupted the 5' part of the gene. The rearrangements caused by the *P*-element insertions were initially characterized by Southern blotting and subsequently DNA flanking the *P*-element insertions was amplified by PCR. Sequence analysis of the amplified DNA enabled us to determine the precise site of integration of the *P*-element. In one case (allele *NF1*^{P1}), the *P*-element insertion has been accompanied by a deletion event that has removed all but the first exon of the *NF1* gene. The 3' end of the deletion is the site of integration of the original *P*-element in the K33 line. In the second mutant allele (*NF1*^{P2}), the *P*-element has integrated in the first intron of *NF1* in the absence of any obvious additional rearrangements.

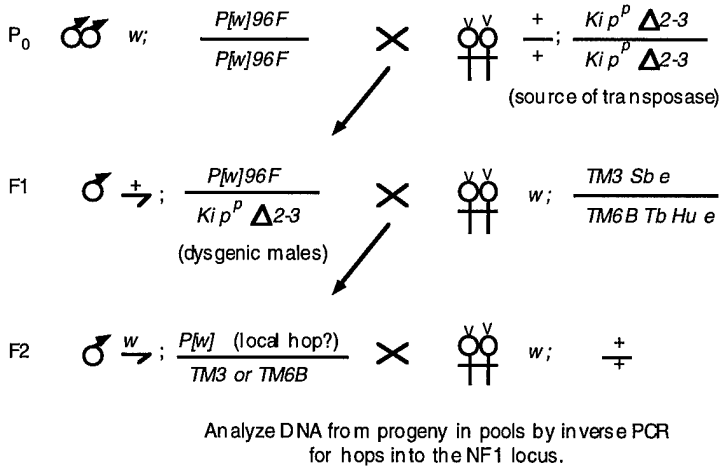

Figure 4. Scheme for the generation of "local hops"

Both mutations are viable when homozygous. However, the *NF1* homozygotes are significantly (~20-30%) smaller than wild-type flies during all post-embryonic stages of the life cycle. Furthermore, each mutation has the same phenotype in trans to a deletion of the region as when homozygous and hence behaves as a null by genetic criteria. Since $NF1^{P1}$ has deleted virtually all of the *NF1* gene, it clearly represents a null allele. This argues strongly that the *NF1* gene is not essential for viability. Since both mutations are viable when homozygous, we prepared extracts from homozygous mutant embryos and examined them by Western blotting using the monoclonal antibodies generated against the C-terminal part of the protein. As shown in Figure 5, no *NF1* protein, which migrates at approximately 280 kDa is detectable in embryonic extracts prepared from either $NF1^{P1}$ or $NF1^{P2}$ embryos. This finding coupled with the observation that for either allele the hemizygous phenotype is the same as the homozygous phenotype indicates that both alleles are likely to represent a complete loss of function.

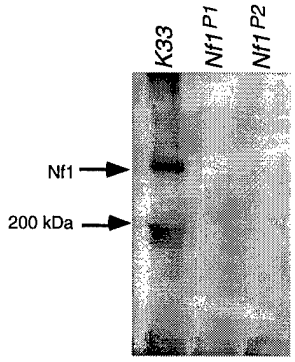

Figure 5: Lack of *NF1* protein in homozygous $NF1^{P1}$ and $NF1^{P2}$ embryos

Analysis of *NF1* mutants

NF1 mutants are smaller than wild type animals as larvae, as pupae and as adults (Figure 6b and not shown). Since a reduction in size might reflect a reduction in cell size or a reduction in cell numbers, we compared the wings of wild type and mutant animals. The linear dimensions of wings of *NF1* mutants are 25-30% smaller than those of wild type flies (Figure 6c and d). Since each wing epidermal cell secretes a single hair, cell densities can be determined by counting the number of hairs in a defined region. Both homozygous *NF1* mutants had a 25-30% higher wing epidermal cell density compared to the parental line (Figure 6e). However, the same is not true in other tissues, since the eyes of *NF1* mutants show a reduced number of ommatidia of normal size and structure (Figure 8 and not shown). Similarly, *NF1* deficient embryos are not reduced in size (not shown).

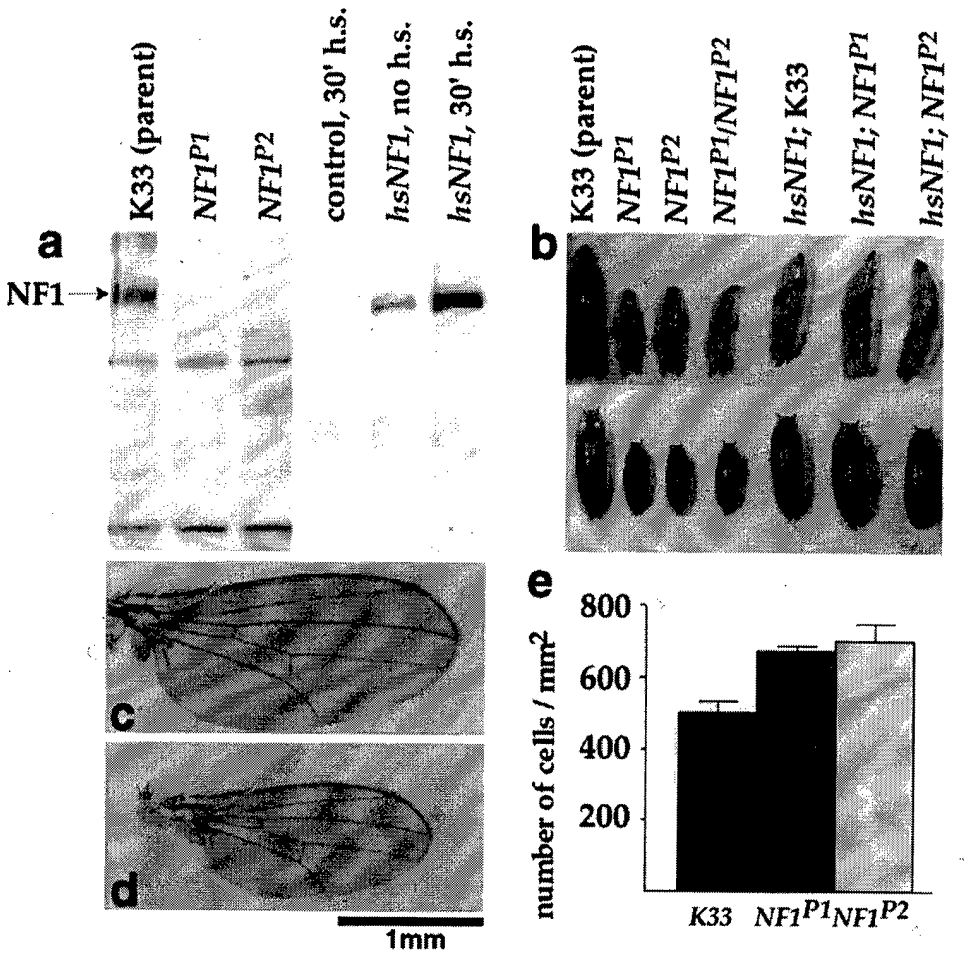

Figure 6. *NF1* mutants exhibit a size defect which is rescued by expression of a *hsNF1* transgene, and which in wings reflects a reduced cell size. Panel A shows immunoblots of embryo extracts probed with monoclonal antibody DNF-21. The arrow indicates an immunoreactive protein migrating around 280 kDa that is present in the K33 parental strain, but absent in either $NF1^{P1}$ or $NF1^{P2}$. A transgenic strain harboring a second chromosome *hsNF1* transgene significantly over-expressed a 280 kDa immunoreactive protein even without heat shock induction (right lanes, panel A). The shorter exposure time makes the endogenous protein hard to detect in the transgenic blot. Panel B shows wandering third instar larvae (top) and pupae (bottom) of the indicated genotypes. The *hsNF1* strain was heat shocked at 37° C for 30 minutes daily. Panels C and D show wings from K33 and $NF1^{P1}$ flies, respectively. The graph in panel E shows the number of wing epidermal cells/mm^2 in the indicated strains.

To determine whether the reduced size of wing epidermal cells reflected a cell autonomous defect, we used X-irradiation to induce mitotic recombination in the wings of heterozygous *NF1* mutants, using the markers *bold* and *forked* to distinguish homozygous mutant clones from surrounding tissue which was heterozygous for *NF1*. No difference in the distance between wing hairs was observed between multiple $NF1^{-/-}$ clones and surrounding cells (Figure 7). Thus the reduced size of wing epidermal cells reflects a non-cell autonomous requirement for *NF1*.

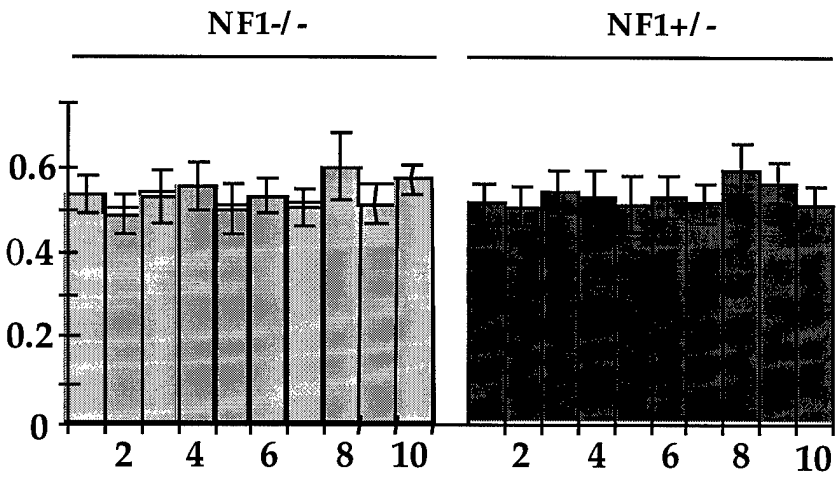

Figure 7: Distances between adjacent wing hairs (in mm). 30 inter-hair distances were scores in each mutant clone as well as in an adjacent heterozygous area.

NF1 mutants also display a subtle behavioral defect characterized by a diminished escape response. Thus, in an assay that determined the number of flies that fly away within 90 seconds of release or after repeated gentle agitation at 15 second intervals between 13 to 16% of either *NF1* mutant (n=200) failed to respond, as compared to 3% non-responders for the parental K33 strain. This reduced escape rate does not reflect obvious anatomical defects of the peripheral nervous system or the musculature, and the mutants scored within normal parameters in assays which measured their activity or their response to visual or olfactory stimuli. In a collaborative effort with the laboratory of Dr. Yi Zhong (Cold Spring Harbor Laboratorye), w demonstrated that neuropeptide-stimulated synaptic transmission is abnormal in *NF1* mutants, which may explain the diminished escape response (see below).

Interaction of *NF1* with the Ras-mediated signaling pathway

Since mammalian neurofibromin is considered to be an important negative regulator of Ras *in vivo*, we examined for abnormalities in *Ras1*-mediated signalling. Surprisingly, several *Ras1*-mediated signalling pathways appear to function normally in *NF1* mutants. The pattern of *tailless* expression (15) is normal (Figure 8a and b), indicating that *torso*-mediated signalling is not perturbed. To test for abnormalities in *sevenless* signalling (16, 17), we examined the retinas of mutant animals. In homozygotes of $NF1^{P1}$, which also lack part of the neurogenic *Enhancer of split* complex, 25% of ommatidia have one or more additional photoreceptor cells (not shown). However, this phenotype is probably due to loss of some of the *Enhancer of split* transcripts, since the retinas of $NF1^{P2}$ homozygotes, of $NF1^{P2}/NF1^{P1}$ and of $NF1^{P2}/Df(3R)boss^{15}$, a deficiency that uncovers the *NF1* locus, are completely wild type (shown for the $NF1^{P2}$ homozygote in Figure 8c). A particularly sensitive indicator of *sevenless* pathway function is the sev^{E4}; Sos^{JC2}/+ mutant combination (18). Only 16.4% of ommatidia in this double mutant have R7 cells and this number is very sensitive to alterations in the gene dosage of *Ras1* pathway components. Flies of this genotype which are also heterozygous for $NF1^{P2}$ have no significant alteration in the

percentage of R7 containing ommatidia (not shown). Thus two well defined *Ras1*-mediated signalling pathways are not particularly sensitive to *NF1* gene dosage.

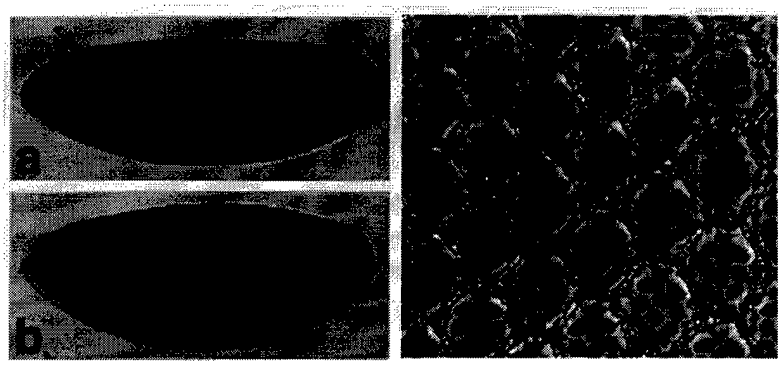

Figure 8: *torso*- and *sevenless*-mediated signalling is not affected by loss of *NF1*. Panels A and B show *tailless* expression detected by RNA *in situ* hybridization in K33 and $NF1^{P2}$ embryos, respectively. Panel C is a retinal section from a $NF1^{P2}/NF1^{P2}$ adult, showing regularly spaced ommatidia of normal size with the normal complement of photoreceptor and accessory cells.

To test whether *NF1* might have a redundant function in regulating *Ras1*-mediated signalling, we attempted to generate flies that were mutant for both *NF1* and for another *Drosophila* RasGAP homologue, *Gap1* (7). Five tested viable heteroallelic combinations of four *Gap1* alleles ($Gap1^{A13p}$, $Gap1^{Ae-1}$, $Gap1^{Ae-2}$, $Gap1^{3ij}$) were each lethal in combination with loss of *NF1* function. Expression of *hsNF1* rescued this lethality (not shown). Thus while either RasGAP is not essential for viability, loss of both is lethal, arguing that these proteins have redundant functions in regulating at least one essential signalling pathway.

The size defect of *NF1* mutants may reflect the inability of other RasGAPs to compensate for loss of *NF1* in regulating a particular *Ras1*-mediated pathway. If so, then reducing the gene dosage of *Ras1* pathway components may influence this phenotype. However reducing the dosage of *Ras1* or of *Sos* by 50% had no effect on the size of *NF1* mutant pupae, nor did crossing in an activated Raf^{gof} mutation (19)(Figure 9). Neither reducing nor increasing signalling through the *Ras1-Raf* pathway therefore modifies the *NF1* phenotype. Since human neurofibromin can also stimulate the GTPase activity of R-Ras, we also tested the effects of reducing the dose of the *Drosophila* R-Ras homologue, *Ras2*. Once again no effect was observed (not shown). Thus, the small size phenotype may result from a property of *NF1* independent of its function as a GAP for Ras-like GTPases.

Interaction of *NF1* with PKA-mediated signaling

Since among RasGAPs neurofibromin is most closely related to IRA proteins, which in yeast function in a pathway in which Ras couples to adenylyl cyclase, and because *Ras1* and cAMP-dependent synaptic signalling is defective in *Drosophila NF1* mutants (accompanying paper), we tested whether cAMP-mediated signalling might represent an alternate target for *NF1* function. *NF1* mutants that were also heterozygous for two different loss-of-function mutations in the protein kinase A (PKA) catalytic subunit homologue *DCO* did not show any alteration in their phenotype (20). However, the small

size of *NF1* mutants was largely rescued by crossing in a constitutively active murine PKA* transgene (21)(Figure 9). Heat shock induction of this constitutively active PKA mutant resulted in lethality. However, significant rescue of the pupal size defect was already observed in cultures grown at 25° C, presumably reflecting leaky transgene expression. As for the size defect, the neurotransmission defect is also rescued by manipulating cAMP levels (see below). This indicates that activation of PKA can bypass a complete loss of *NF1* function in at least two different situations *in vivo*.

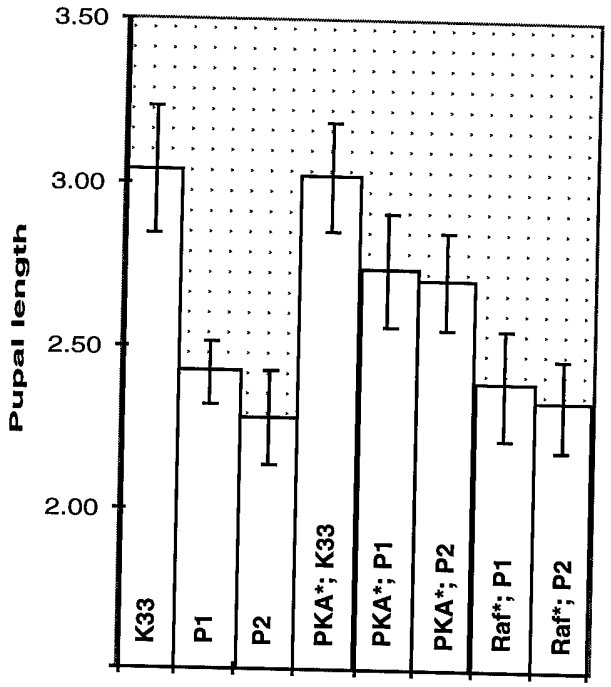

Figure 9: Rescue of the pupal size defect of *NF1* mutants by *hsNF1* and activated PKA, but not by activated *raf*. The graph presents the average length of pupae plus or minus the standard deviation. The genotypes analyzed were: **1**; K33/K33 (parent stock) **2**; $NF1^{P1}/NF1^{P1}$. **3**; $NF1^{P2}/NF1^{P2}$. **4**; *hsp70-PKA**; K33/K33. **5**; *hsp70-PKA**; $NF1^{P1}/NF1^{P1}$. **6**; *hsp70-PKA**; $NF1^{P2}/NF1^{P2}$. **7**; *hs-raf**; $NF1^{P1}/NF1^{P1}$. **8**; *hs-raf**; $NF1^{P2}/NF1^{P2}$. Between 50 and 90 pupae were measured for each genotype.

Role of *NF1* in synaptic neurotransmission

A potential explanation for the abnormal behavior of the *NF1* mutants is that neuromuscular function is abnormal. This seems especially likely in view of the recent observation by Dr. Yi Zhong (Beckman Center for Memory and Learning, Cold Spring Harbor Laboratory), that Ras1 and Raf play a crucial role in synaptic transmission at the larval neuromuscular junction. Dr. Zhong has demonstrated that a specific synaptic current at the neuromuscular junction requires the function of both Ras1 and Raf (22).

At the neuromuscular junction of the larval body wall, a neuropeptide related to the mammalian pituitary adenyl cyclase-activating polypeptide (PACAP38) functions as a neurotransmitter. Application of PACAP38 simulates the effects of high frequency stimulation of the motor axons. This leads to a biphasic response; a slow inward current which leads to a depolarization of the muscle membrane and subsequently a 100-fold increase in voltage-activated K^+ conductance (Figure 10). The inward current leads to depolarization of the muscle membrane. The subsequent enhancement of K^+ conductance probably functions either to retard the generation of action potentials or to shorten the duration of the action potential.

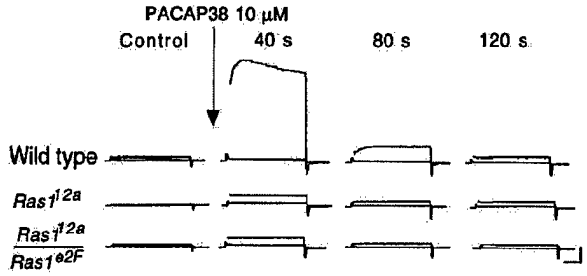

Figure 10. Electrophysiological tracings showing K^+ conductance changes after PACAP38 stimulation in wild type and *Ras1* mutant larvae. See (22) for details.

In larvae that are mutant for either *Ras1* (Figure 10) or *Raf*, the enhancement of the K^+ conductance is undetectable following the addition of 1mM or 10 mM of PACAP38. By examining the currents in larvae bearing gain-of-function Raf alleles, it was demonstrated that stimulation of the Ras1/Raf pathway is necessary but not sufficient for the K^+ current. Similarly, mutants in the cAMP pathway abolish the K^+ current but the application of cAMP analogs alone is insufficient to generate the current. Activation of both the Ras1/Raf pathway and the application of cAMP analogs is sufficient to generate the current. This suggests that signaling via both pathways is both necessary and sufficient for generating the K^+ current (Figure 11).

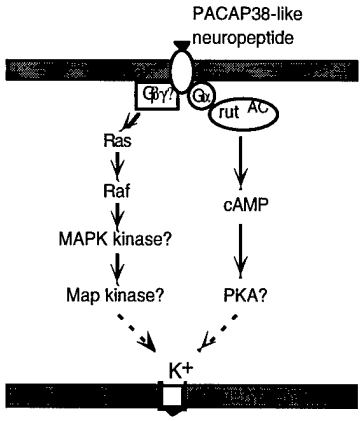

Figure 11: Proposed model for regulation of post-synaptic potential

In collaborative experiments with Dr. Zhong's laboratory, we have shown that the enhancements of K^+ conductance is completely abolished in both mutant alleles of *NF1*. This defect is completely rescued by crossing in an *NF1* transgene under the control of the inducible *hsp70* promoter. Application of two different membrane-permeable cAMP analogs, dibutyryl cAMP or 8-bromo-cAMP to the larval neuromuscular preparation restores the normal response to PACAP38. cAMP anlogs were effective if applied anytime prior to and within two minutes of applying PACAP38. To further test whether activation of cAMP signaling rescues the defective PACAP response of *NF1* mutants, the drug forskolin, which stimulates G-protein coupled adenylyl cyclase activity was applied to the neuromuscular preparation. PACAP38 induced a normal response in *NF1* mutants after forskolin incubation, similar to the effect of cAMP analogs.

Thus, as with the size defect, the defect in neuromuscular transmission can be rescued by activating the PKA pathway - in this case pharmacologically.

DISCUSSION

We have cloned and characterized a highly conserved Drosophila *NF1* homologue. The high degree of sequence conservation throughout the protein as well as the conserved location of intorns in the gene argue that it is indeed a true homologue. The lack of evidence for any other *NF1*-like genes in Drosophila despite screening by low-stringency hybridization is indicates that this gene is likly to represent the only *NF1* gene in Drosophila. Analysis of the expression of *NF1* RNA and protein demonstrated that the gene was expressed in most, if not all, tissues throughout development arguing that *NF1* is unlikely to be a tissue specific signaling protein. In particular we found no evidence that *NF1* expression was higher in neural tissues.

At the onset of this study, we considered it likely that mutations in Drosophila *NF1* were likely to be lethal. This was based on the evidence that mutations in *NF1* in mice cause lethality during embryonic development (23, 24). We therefore saturated the region spanning the *NF1* locus for lethal mutations. However, to our surprise, none of the lethal mutations corresponded to *NF1* mutations. We subsequently isolated mutations in *NF1* by mobilizing P-element transposons and screening by PCR for insertions in the *NF1* locus. Analysis of the mutant alleles confirmed that complete loss of function mutations are indeed viable. Thus mutations in Drosophila *NF1* do not appear to compromise a number of Ras1-mediated signaling pathways that are essential for normal development and viability. We don not understand why mutations in murine *NF1* are lethal while mutations in Drosophila *NF1* are not. One possiblity is that many of the essential functions of Drosophila *NF1* can be carried out by other RasGAPs in vivo. In support of this hypothesis is our finding that the double mutant combination of *NF1* and *Gap1* is lethal in Drosophila.

Our studies demonstrate that *NF1* is not a crucial and global negative regulator of *Ras1* function in vivo. We tested for the efficacy of *Ras1*-mediated signaling in a variety of genetic backgrounds which are extremely sensitive to variations in signaling strength. We were unable to find any evidence of abnormalities in either *torso* or *sevenless* signaling in *NF1* mutants implying that *NF1* does not modulate either of these pathways significantly in vivo. Our future studies will attempt to analyse the function of these pathways in double mutant combinations (mutations in *NF1* and other RasGAPs).

We have shown that *NF1* is necessary for normal growth and for normal neuromuscular function *in vivo*. This study represents the first demonstration that *NF1* is required for either of these processes. Since the effect on growth of the wing is non cell

autonomous, *NF1* may be necessary in a group of cells that regulates the growth of the organism. These may be cells that secrete one of the hormones that regulates the growth of Drosophila. In the future, we propose to use the UAS-GAL4 system (25) to map the cells that require *NF1* in vivo for normal growth regulation.

By far the most novel finding of our studies is the demonstration of cross-talk between *NF1* and the PKA-mediated signaling pathway. We have been able to demonstrate this phenomenon in two completely different experimental contexts. Firstly, we were able to show that the growth defect could be significantly suppressed by crossing in a constitutively active PKA subunit. Secondly, the neuromuscular defect could be corrected by pharmacological activation of the PKA pathway. We presently do not understand the precise nature of the interaction between *NF1* and PKA. Since activation of PKA can suppress a phenotype resulting from a complete loss of *NF1* function, PKA cannot function upstream of *NF1*; PKA must function either downstream of *NF1* or in a parallel pathway. Our future studies will employ techniques such as the yeast two hybrid system, which can identify protein-protein interactions to test potential interactions between components of the PKA pathway and *NF1*.

Since both the *Ras* and PKA pathways are highly conserved between *Drosophila* and humans, it is likely that human *NF1* will also interact with PKA-mediated signaling. While caution is necessary in extrapolating results obtained in Drosophila to a vertebrate context, most signaling pathways studied in Drosophila appear to be essentially identical to their vertebrate counterparts. If so, agents which increase PKA activity may eventually play a role in the treatment of human type 1 neurofibromatosis.

CONCLUSIONS

1) Drosophila has a highly conserved *NF1* homolog
2) Drosophila *NF1* is expressed at most, if not all, stages of development in most tissues
3) Loss of function mutations in *NF1* are not lethal but cause lethality in combination with mutations in Gap1
4) Many Ras1-mediated signaling pathways function normally in *NF1* mutants
5) *NF1* mutants show defects in organismal growth and in neuromuscular transmission
6) Both defects can be suppressed by activation of the PKA pathway

References

1. A. Bernards, *Biochim. Biophys. Acta* **1242**, 43-60 (1995).
2. D. L. Lindsley, G. G. Zimm, *The genetics and biology of Drosophila melanogaster* (Academic Press, San Diego, 1992).
3. K. Basler, B. Christen, E. Hafen, *Cell* **64**, 1069-1081 (1991).
4. W. R. Engels, C. R. Preston, P. Thompson, W. B. Eggleston, *Trends Genet* **5**, 366 (1989).
5. D. Tautz, C. Pfeifle, *Chromosoma* **98**, 81-5 (1989).
6. M. Ashburner, *Drosophila: A laboratory Manual.* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989).
7. U. Gaul, G. Mardon, G. M. Rubin, *Cell* **68**, 1007-1019 (1992).
8. J. G. Bishop III, V. G. Corces, *Genes and Dev.* **2**, 567-577 (1988).
9. B. A. Edgar, G. Schubiger, *Cell* **44**, 871-877 (1986).
10. M. Frasch, M. Levine, *Genes Dev.* **1**, 981-995 (1987).
11. I. K. Hariharan, et al., *EMBO J.* **14**, 292-302 (1995).
12. A. C. Hart, H. Kramer, D. L. Van Vactor, M. Paidhungat, S. L. Zipursky, *Genes and Devel.* **4**, 1835-1847 (1990).

13. E. Knust, K. Tietze, J. A. Campos-Ortega, *EMBO J.* **13**, 4113-4123 (1987).
14. B. Dalby, A. J. Pereira, L. S. B. Goldstein, *Genetics* **139**, 757-766 (1995).
15. J. B. Duffy, N. Perrimon, *Dev. Biol* **166**, 380-395 (1994).
16. B. Dickson, E. Hafen, *Curr. Opin. Genet. Dev.* **4**, 64-70 (1994).
17. M. A. Simon, D. D. L. Bowtell, G. S. Dodson, T. R. Laverty, G. M. Rubin, *Cell* **67**, 701-716 (1991).
18. R. D. Rogge, C. A. Karlovich, U. Banerjee, *Cell* **64**, 39-48 (1991).
19. A. H. Brand, N. Perrimon, *Genes Dev.* **8**, 629-639 (1994).
20. M. E. Lane, D. Kalderon, *Genes Dev* **7**, 1229-43 (1993).
21. J. Jiang, G. Struhl, *Cell* **80**, 563-572 (1995).
22. Y. Zhong, *Nature* **375**, 588-592 (1995).
23. C. I. Brannan, et al., *Genes Dev.* **8**, 1019-1029 (1994).
24. T. Jacks, et al., *Nature Genetics* **7**, 353-361 (1994).
25. A. H. Brand, N. Perrimon, *Development* **118**, 401-415 (1993).

Publications from this effort

1) The, I., Hannigan, G. E., Reginald, S., Zhong, Y., Gusella, J. F., Hariharan, I. K. and Bernards, A. Role for Drosophila *NF1* in growth regulation and PKA-mediated signalling. (submitted to Nature)

2) Guo, H-F., The, I., Hannan, F., Hariharan, I. K., Bernards, A and Zhong, Y. neurofibromin regulated signaling in Drosophila neuropeptide transmission: adenylyl cyclase and ras pathways. (submitted to Nature)

Meeting Abstracts

Inge The, Andre bernards and Iswar K. Hariharan. Analysis of the function of the Neurofibromatosis gene of Drosophila melanogaster. Molecular Neurobiology of Drosophila. October 5-October 9, 1995, Cold Spring Harbor Laboratory, New York.

Inge The, Gregory Hannigan, Yi Zhong, Iswar Hariharan, Andre Bernards. Analysis of *NF1* gene function in Drosophila Cancer Genetics & Tumor Suppressor Genes. August 14-18, 1996, Cold Spring Harbor Laboratory, New York.

S.I. The, G. Hannigan, S. reginald, J. F. Gusella, I. K. Hariharan and A. Bernards. Characterization of a Drosophila *NF1* homologue and its role in Ras1 mediated signal transduction. 37th Annual Drosophila Research Conference San Diego, California April 27 - May 1, 1996.

Personnel paid from this grant

1) Iswar K. Hariharan	P.I.
2) Andre Bernards	co-P.I.
3) S. Reginald	research technician
4) A. J. Snijders	graduate student/technician
5) S. Inge The	graduate student/technician
6) S. Brill	post-doctoral fellow
7) K. Graber	research technician

Only Drs. Hariharan and Bernards were funded for the full duration of the grant. Two other personnel were funded from this source at any time. Ms. The replaced Ms. Reginald and Dr. Brill replaced Dr. Snijders. Ms. Graber was hired during the last 5 months of the funding period to replace Dr. Brill.